Zootaxa 4656 (3): 535–544
https://www.mapress.com/j/zt/

Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

https://doi.org/10.11646/zootaxa.4656.3.10
http://zoobank.org/urn:lsid:zoobank.org:pub:E236AFC9-76EA-4F64-8045-268C5417E46A

Acricotopus indet. morphotype incurvatus: Description and genetics of a new Orthocladiinae (Diptera: Chironomidae) larval morphotype from the Tibetan Plateau

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Abstract

High mountain ranges such as the Tibetan Plateau with an average altitude above 4500 m are topographically complex formations. Elevational gradients, physiographic diversity and climatic heterogeneity have led to highly biodiverse ecosystems in these regions. Mountain ranges can be seen as cradles of evolution and harbour, due to their unique characteristics, a high number of highly adapted species. At the same time these areas are hard to access and therefore taxonomic information is limited. Here we describe a new *Acricotopus* (Diptera: Chironomidae: Orthocladiinae) larval morphotype occurring in lakes and ponds of differing salinity and water depths located on the Southern and Central Tibetan Plateau. The description is based on larvae and their genetics (ribosomal 18S, 28S and mitochondrial COI sequences) collected from a shallow pond in close proximity to the large saline lake Selin Co. Larvae of *Acricotopus* indet. morphotype incurvatus are characterized by a mentum with a cluster of lateral teeth, partially folded inwards, a mandible with a toothed lobe in addition to four inner teeth and a sclerotized plate positioned behind the mentum. Up to now, these morphological features have only been found in early instars of other *Acricotopus* species. The proposed morphotype name is inspired by the peculiar form of the mentum.

Key words: Chironomidae, Orthocladiinae, *Acricotopus*, systematics, morphotype, larva, DNA barcoding, Tibet, Central Asia

Introduction

Acricotopus (Diptera: Chironomidae: Orthocladiinae) is a widespread genus with Holarctic and Oriental distribution, with five species described from the Palaearctic (Andersen *et al.* 2013), two from Nearctic and two from Oriental (Ashe & O'Connor 2012). Larvae of *Acricotopus* are common in small bodies of standing and slow-flowing waters, temporary bodies of still water, and in lake littorals (Andersen *et al.* 2013). Subfossil larval head capsules of *Acricotopus* indet. morphotype incurvatus have previously been found in several independent studies on the Tibetan Plateau, where they were initially listed as Orthocladiinae indet. (Hamerlík *et al*. 2010), *Acricotopus* indet. 1 (Plank 2010) and later under the codename of Orthocladiinae type K (Laug *et al*. 2018). Here we present a morphological

Accepted by B. Rossaro: 15 Jul. 2019; published: 15 Aug. 2019

description of a previously undescribed larval morphotype, *Acricotopus* indet. morphotype incurvatus, based on morphological characteristics as well as DNA material derived from living larvae collected from a small pond on the Tibetan Plateau. Morphological distinction from other genera as well as other *Acricotopus* species is primarily based on the shape and arrangement of the larval mentum and mandible as well as the presence of a sclerotized plate, situated behind the mentum, which is otherwise only known from one species of a different subfamily, *Tanytarsus gracilentus* Holmgren (Chironominae: Tanytarsini). While these characteristics suffice to distinguish it from other described larvae, the comparison to *Acricotopus simplex* Zhang and *A. zhalingensis* Zhang has not been possible, because only morphological information on the male adults exist. *Acricotopus* indet. morphotype incurvatus larvae occur in high elevation lakes and ponds on the Tibetan Plateau, from fresh- to saline and shallow (less than 50 cm water depth) to deep (35 m water depth) water bodies.

Methods

Seventeen specimens were collected from a small pond situated a few meters away from the second largest lake of the Tibet-Qinghai Plateau, Selin Co (31.728468°N, 88.854926°E). Sediment-, kicking-, floating- and plant-samples were collected using a 250 µm mesh sized net during field work in July and August 2018. The floating samples were sorted directly at the sampling site, all other samples after transportation and preparation. All samples have been stored in 96 % ethanol prior to preparation. Six specimens have been mounted in Berlese medium following the separation of the head capsules from the body as well as treatment with 10 % caustic potash (KOH) for two hours at room temperature. Terminology follows Andersen *et al*. (2013) except where larval features follow Brooks *et al*. (2007). All specimens are deposited at the Beijing Museum of Natural History, P. R. China.

Genomic DNA of the specimens (n=5) was extracted using DNeasy Blood & Tissue Kit (QIAGEN, Germany) following the manufactures instructions. The cytochrome oxidase subunit 1 (COI) region was amplified using the primers LCO1490 and HCO2198 (Table S1; Folmer *et al*. 1994). Fragments of the small and large subunit of ribosomal RNA (18S and 28S) were amplified using primer pairs 18S_ai-18S_bi and S3660-A335 (Table S1; Whiting *et al*. 1997; Morse & Normark, 2006), respectively. The PCR mixture contained 0.5 µl of each primer (10 µM), 5 µl 5x HOT FIREPol® Blend Master Mix (Ready to Load, with 10 mM $MgCl_2$, Solis BioDyne, Estonia), 1 µl of DNA template and 18 µl of sterilized distilled water. PCR conditions for COI region amplification consisted of 15 min at 95 ºC, five cycles of 1 min at 94 ºC, 1 min at 45 ºC, and 1 min at 72 ºC, 35 cycles of 1 min at 94 ºC, 1 min at 51 ºC, and 1 min at 72 ºC, and 5 min at 72 ºC. For both, 18S and 28S regions, the amplification cycles consisted of initial denaturation at 95 ºC for 15 min, 30 cycles of 95 °C for 30 s, decreasing temperature by 2 °C every 6th cycle from 57 °C to a touchdown at 47 °C for 60 s, 72 °C for 1 min and final extension at 72 °C for 10 min.

Amplification success was checked with 1 % agarose gel via electrophoresis. PCR products were cleaned using Exo-TSAP enzymes using incubation at 37 °C for 15 min and at 85 °C for 15 min. PCR products were sequenced in LGC, Biosearch Technologies (Berlin, Germany) using the PCR primers. Quality check and manual editing was performed using CodonCode Aligner (v8.0.2; CodonCode Corp., USA). All sequences are deposited in plutoF database (Abarenkov *et al*. 2010) and GenBank (Benson *et al.* 2008; accession numbers in Table S2); submission pending. All sequences were verified to belong to Chironomidae using the blastn algorithm against GenBank.

Visualizing the placement of the studied specimens in a phylogenetic tree, we used our generated data alongside with additional 18S and 28S sequences from Cranston *et al*. (2012), *Acricotopus lucens* Zetterstedt reads from Staiber (2004; Table S2) and *Acricotopus longipalpus* Reiss Tang *et al.* (2019; BOLD Sample ID: ACRIC-2L-1M). Sequences were aligned using online MAFFT (v7; Katoh *et al*. 2017) with default settings and edited in Seaview (v4.7; Gouy *et al*. 2010). A maximum likelihood tree was constructed using RaxML (v8.2.12; Stamatakis, 2014) with GTRGAMMA substitution model and 100 bootstraps.

Taxonomy

Acricotopus indet. morphotype incurvatus

(Figure 1–3)

Orthocladiinae type K3 (Laug *et al*. 2018)

Type Material. [all Beijing Museum of Natural History, P.R. CHINA: Tibet, Nakchu Province, 31.728468°N, 88.854926°E, 02.08.2018, A. Laug, slides mounted in Berlese medium]: Holotype Larva1, 4th instar, , BMNHC239441, Holotype Larva 2, 3rd instar, BMNHC239440; Holotype Larva 3, 3rd instar, BMNHC239442; Holotype Larva 4, 2nd instar, BMNHC239443; Holotype Larva 5, 4th instar, anterior part of body missing, BMNHC239444; Holotype Larva 6, 3rd instar, body missing, BMNHC239445.

Diagnosis: Larva. Medium-sized larvae (5.7 mm body length), head capsule length 0.5 mm and about as long as wide, head coloration brown with occipital margin dark brown to black, wrinkles might be present, pale spot on the frons. This characteristic is not always visible in weakly sclerotized specimens.

Antenna. With 5 segments, about 1/6 to 1/7 as long as head capsule length, basal segment longer than flagellum, ring organ and basal setae close to the base, antennal segment 2 about as long as combined length of segments 3–5. Blade shorter than flagellum, reaching until the end of the 4th segment, Lauterborn organs indistinct. Style as long as the 3rd segment.

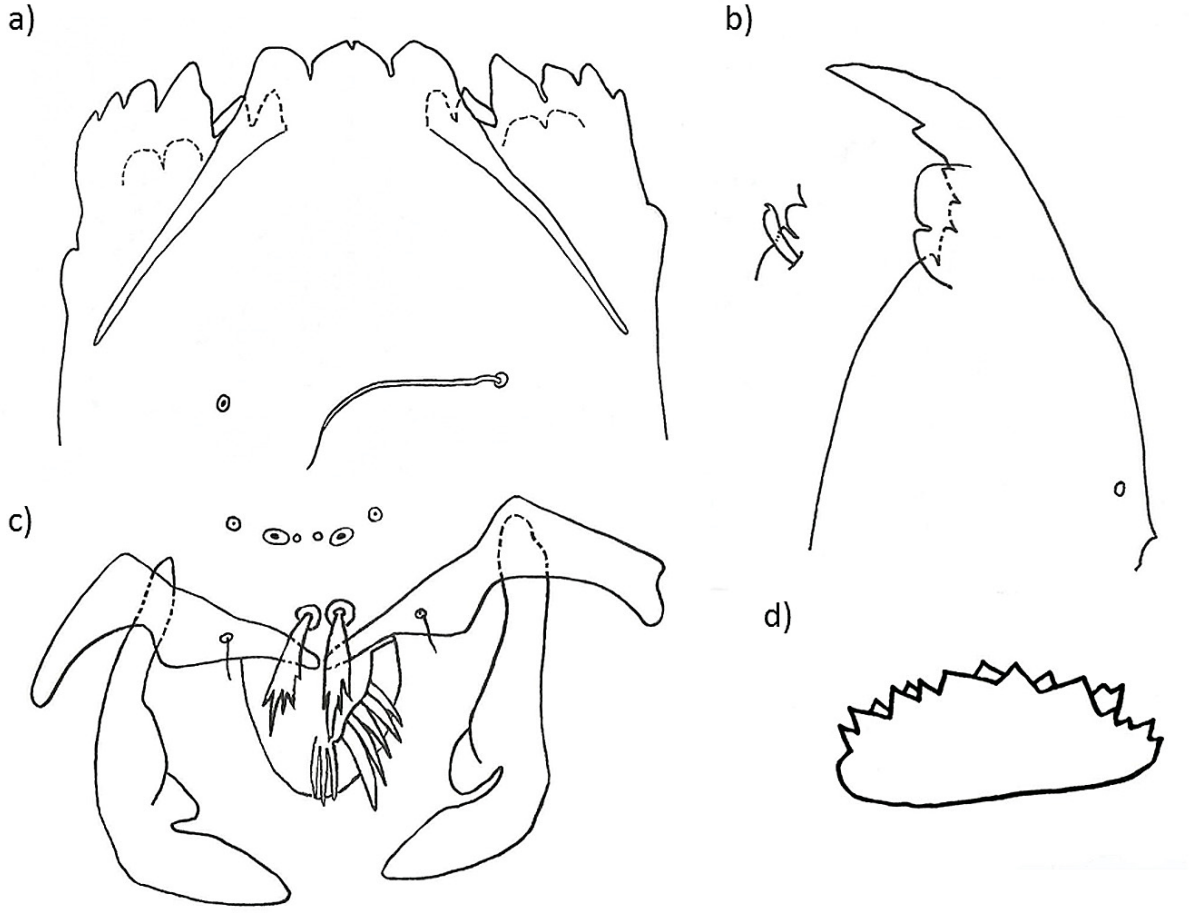

FIGURE 1. *Acricotopus* indet. morphotype incurvatus 4th instar larva. a) Mentum. b) Mandible with seta subdentalis. c) Labrum with premandibles, S I and pecten epipharyngis. d) Sclerotized plate.

Labro-epipharyngeal region. S I palmate, with 4–5 lobes. S II and S III simple, pecten epipharyngis with 3 lobes, premandible with one apical and one basal tooth, brush absent.

Mandible. Apical tooth about as long as combined width of 4 inner teeth, one toothed lobe, subdivided into 2 parts, the length varying considerably between shorter than the margin of the mandible to overlying the inner teeth. Seta subdentalis notched with a hook, seta interna absent.

Mentum. With 3 median teeth, middle median tooth subequal to outer median teeth and can be notched. Up to 8 lateral teeth on a different focal plane than median teeth, first 2 lateral teeth reduced and on different focal plane than other teeth, partially behind of median and outer lateral teeth, 3rd to 8th lateral tooth in a distinct cluster and curved inwards. The 7th and 8th lateral tooth behind 3rd to 6th lateral tooth, because of the dark coloration of the mentum often not visible. Ventromental plate narrow anteriorly, extending into sclerotized, rounded plate beyond basal

margin of mentum. Sparse beard with up to 10 is present even if reduced in comparison with the other *Acricotopus* described species, setae submenti positioned between ventromental plates. Large narrow plate, similar to the one of *Tanytarsus gracilentus*, with 4–6 teeth on each of two focal planes, positioned behind mentum.

Maxilla. Pecten galearis absent, setae maxillaris simple, maxillary palp slightly longer than wide.

Body. White, when stored in alcohol with blue coloration at the dorsal side between segments. Anterior parapods separate, each bearing apical crown of claws, sometimes claws with a distinct apical tooth. Posterior parapods separate, each with an apical group of simple claws, procercus about as high as wide, dark, strongly sclerotized, bearing 6 anal setae, anal tubules much shorter than posterior parapods.

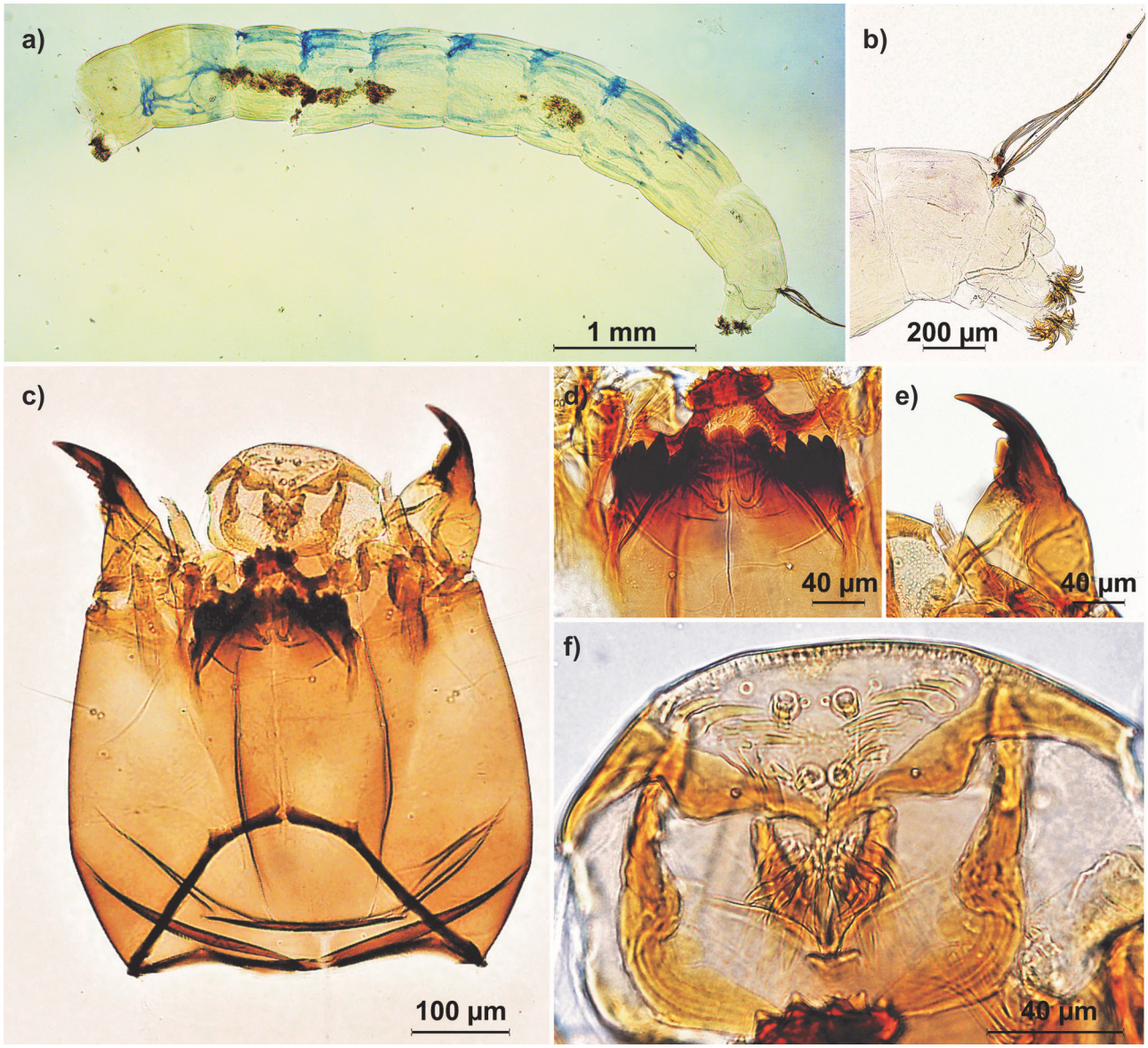

FIGURE 2. *Acricotopus* indet. morphotype incurvatus 4th instar larva, light microscopy pictures. a) Body without head capsule. b) Last abdominal segment with posterior parapods, anal tubules and anal setae. c) Head capsule. d) Mentum with sclerotized plate. e) Mandible. f) Labro-epipharyngeal region.

Genetics. A total of five 28S, five COI high quality sequences and one 18S read were obtained for the studied specimens. Reads of 28S and COI did not exhibit any intraspecific sequence divergence. Lengths of the *Acricotopus* indet. morphotype incurvatus sequences used in the construction of maximum likelihood tree were 956 and 656 for 18S and 28S, respectively. In the phylogenetic tree, *Acricotopus* indet. morphotype incurvatus placed alongside other Orthocladiinae, with strong support values (Fig. 4). However, as we used only 18S and 28S reads for the phylogenetic tree construction, the aim is not to resolve the phylogenetic replacement of the taxa, but to demonstrate the replacement to other Orthocladiinae. Adding COI alignments to the tree construction did not change the placement

of *Acricotopus* indet. morphotype incurvatus, but induced a long branch to this group (data not shown). As the 28S reads were identical between *Acricotopus* indet. morphotype incurvatus specimens, in the tree we used only one specimen from which we were able to obtain also the 18S sequence (Table S2).

In accordance, the closest blastn matches, against GenBank database, were against Orthocladiinae: 18S and 28S matched with 92.54 % and 97.29 % similarity to *Acricotopus lucens* (AJ586561, AJ586562), respectively; COI sequences exhibited 90.05 % to specimen assigned to *Cricotopus* sp. (KM571496, Orthocladiinae). Similarity of *Acricotopus* indet. morphotype incurvatus COI and 28S reads against *A. longipalpus* were 87.2 % and 88.1 % (Levenshtein distance), respectively, and against *A. lucens*, 86.0 % and 96.8 %, respectively. Similarity of *Acricotopus* indet. morphotype incurvatus 18S sequences against *A. lucens* was 92.0 % (no data for *A. longipalpus* 18S).

Etymology. The morphotype name "incurvatus" is inspired by the peculiar form of the larval mentum.

Remarks. Gut content: Detritus including inorganic particles like fine sand, and organic remains such as diatoms and other algae.

At the sample point SLChS 18-04 larvae have been found in sediment-, kicking- and floating-samples, but were absent from plant-samples.

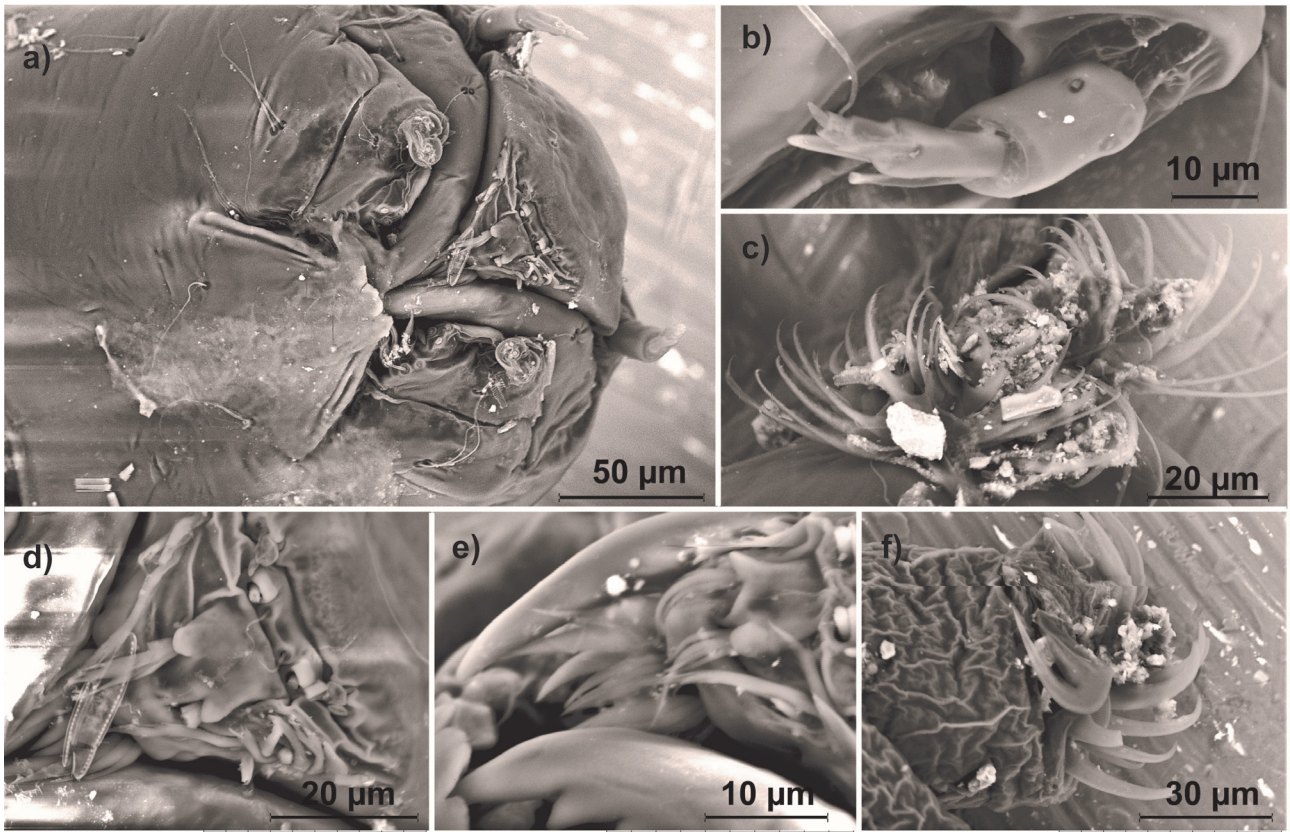

FIGURE 3. *Acricotopus* indet. morphotype incurvatus 4th instar larva, scanning electron microscope pictures. a) Head capsule. b) Antenna. c) Anterior parapods. d) Labro-epipharyngeal region with S I. e) Pecten epipharyngis with S I. f) Posterior parapods.

Discussion

Classification. While some features of *Acricotopus* indet. morphotype incurvatus such as antennae, premandibles, strikingly dark occipital margin and procercus, notched seta subdentalis, and presence of beard (which is however, sparse and not always visible) suit the generic diagnosis of *Acricotopus*, other morphological characteristics deviate from it. Three inner teeth on the mandible are typical for *Acricotopus*, whereas *A.* indet. morphotype incurvatus has 4 inner teeth. At the same time the mandibles feature a large surficial tooth shaped like a toothed lobe which is not present in other *Acricotopus*. The most distinctive feature of *A.* indet. morphotype incurvatus is the folded mentum bearing 3 median and 8 lateral teeth. Moreover, there is a large narrow strongly sclerotized toothed plate situated behind/above the mentum. These features are not present in *Acricotopus*, which typically has a mentum bearing a

broad notched median tooth and 6 lateral teeth. Despite these morphological differences, we placed the new species in the genus *Acricotopus*. Based on the ribosomal 18S, 28S (and mitochondrial COI genes), our studied specimens were placed in Orthocladiinae clade, alongside *Acricotopus lucens* with strong support values (Fig. 4), further suggesting the morphotype placement to *Acricotopus*.

FIGURE 4. Maximum likelihood tree of 18S and 28S sequences, with the small subset chironomid taxa from different subfamilies. The numbers near nodes represent bootstrap supports (100 replications). The taxa in bold represent herein described species, *Acricotopus* indet. morphotype incurvatus. *Forcipomyia brevipennis* Macquart represents the outgroup.

While the larval morphological features of *Acricotopus* indet. morphotype incurvatus differ significantly from the most common species *A. lucens*, they resemble those *A. longipalpus*, originally described as adult male from Nepal (Reiss 1968), including partly overlapping mental teeth and the presence of four instead of three inner teeth on the mandible (Zelentsov 1989). Other characteristics though, primarily the mental teeth featuring three instead of four median teeth in *A. longipalpus* as well as the teeth appearing on three different focal planes in *Acricotopus* indet. morphotype incurvatus, enable the differentiation. The mandible differs as well, featuring a longer apical tooth and a large surficial tooth shaped like a toothed lobe not present in *A. longipalpus*, as well as the sclerotized toothed plate that is present in *Acricotopus* indet. morphotype incurvatus. The genetic data confirms the separation from both species, because the COI sequence similarity of *A. longipalpus* to *Acricotopus* indet. morphotype incurvatus were below 90 % and therefore greatly exceeds the ~4.5 % variability threshold found to be appropriate to delimit non-biting midges (Ekrem *et al.* 2018).

Larvae of *Acricotopus maritimus* Zelentsov, described from the arctic region of Russia (Zelentsov 1993), shows

much more similar mental median teeth, featuring 3 median teeth. The lateral teeth though differ distinctly from *A.* indet. morphotype incurvatus: Only the first lateral tooth is reduced and overlaps with the median teeth, the other five lateral teeth are subequal and on the same focal plane. Furthermore, premandibles have one additional tooth and SII are palmate. Hence, the morphotype described in this paper does not belong to the species *Acricotopus maritimus*.

Besides these, two more *Acricotopus* species are described from the Qinghai province: *A. simplex* and *A. zhalingensis* (Zhang & Wang 2004). Although the occurrence of those two species in the region of our study is likely, because the conditions of the neighbouring Chinese regions Tibet and Qinghai are similar, their description is solely based on morphological characteristics of male adults. Thus, a comparison to our larval morphology and DNA results was not possible. Consequently, we cannot reject nor confirm the affiliation of *Acricotopus* indet. morphotype incurvatus to one of them.

Distribution. Head capsules of *Acricotopus* indet. morphotype incurvatus have been found on the Southern and Central Tibetan Plateau, but the accessibility of many Tibetan lakes is difficult. As a result, the number of chironomid studies in this region is low, and the absence of observations in the northern part of the Plateau could very well be a matter of missing information. *A.* indet. morphotype incurvatus certainly is distributed in the Eastern part of the Central Tibetan Plateau below 4550 m a.s.l. Morphologically slightly different larval head capsules have been found at higher elevation in the western part of the Central Tibetan Plateau as well as the Southern Tibetan Plateau (Hamerlík *et al*. 2010, Laug *et al*. 2018). It is currently unclear if these belong to the same species.

Ecology. *Acricotopus* indet. morphotype incurvatus was originally discovered in two shallow ponds, situated on the Southern Tibetan Plateau, characterized by sandy sediments and sparse aquatic vegetation (Hamerlík *et al*. 2010), a habitat similar to the place where the living larvae described in this study were collected. Even though Selin Co is a much deeper (head capsules were frequent up to 35 m water depth) lake than those studied by Hamerlík *et al*. (2010), it also has a sparse vegetation cover and sandy sediments. Because of the fine sand particles that were found in the gut content, we assume that in these water bodies *A.* indet. morphotype incurvatus lives in or on the sediment. Additionally, larvae were not encountered in the water plant samples while they were found in all other sample types. Thirdly, the genus is absent from sample sites with dense vegetation as well as running water.

Both gut content and habitat preferences suggest that larvae of *Acricotopus* indet. morphotype incurvatus are detritovorous feeding on sediment. This is further supported by morphological similarities to *Corynocera ambigua* Zetterstedt which shows a similar cluster of lateral teeth, as well as to *Tanytarsus gracilentus*, characterized by a similar sclerotized plate situated behind the mentum. Both species are sediment feeders as well (Brodersen & Lindegaard 1999, Ingvason *et al*. 2004).

Acknowledgements

We thank Martin Spies for supporting the idea of and recommendations for publishing the larval description, Hongqu Tang for assisting with his taxonomical and genetical expertise, as well as Peter Cranston for his constructive and critical reviews of the manuscript that significantly improved the paper. We are grateful to Fabio Laurindo da Silva, University of Sao Paulo, for his comments to the latest version of the manuscript. The German Federal Ministry of Education and Research (BMBF) sponsors our work within the joint project "CAHOL - Holozäne Klimaschwankungen in Zentralasien" (FKZ 03G0864C). Field work was funded by the National Natural Science Foundation of China (41571189). Parts of the study was funded by the National Science Centre, Poland, contract no. 2015/19/P/ST10/04048. This project has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No. 665778. Further support for this project has been received from the Climate-KIC program "Pioneers into Practice".

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TABLE S1. Primers used in this study.

Primer	Sequence (5'-3')	Reference
LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer *et al*. 1994
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer *et al*. 1994
18S_ai	CCTGAGAAACGGCTACCACAT	Whiting *et al*. 1997
18S_bi	GAGTCTCGTTCGTTATCGGA	Whiting *et al*. 1997
S3660	GAGAGTTMAASAGTACGTGAAAC	Morse and Normark 2006
A335	TCGGAAGGAACCAGCTACTA	Whiting *et al*. 1997

TABLE S2. GenBank and UNITE database accession number for the herein generated sequences, and GenBank accession numbers for the used sequences for constructing the phylogenetic tree (Figure 4; reads from Cranston *et al*. (2012) and Staiber (2004)).

	Database	28S	18S	COI
Acricotopus indet. morphotype incurvatus (CH2)	GenBank	MN160634		MN164354
	UNITE	UDB0777924		UDB0777923
Acricotopus indet. morphotype incurvatus (CH4)	GenBank	MN160635	MN160663	MN164355
	UNITE	UDB0777926	UDB0777927	UDB0777925
Acricotopus indet. morphotype incurvatus (CH5)	GenBank	MN160636		MN164356
	UNITE	UDB0777929		UDB0777928
Acricotopus indet. morphotype incurvatus (CH6)	GenBank	MN160637		MN164357
	UNITE	UDB0777931		UDB0777930
Acricotopus indet. morphotype incurvatus (CH8)	GenBank	MN160638		MN164358
	UNITE	UDB0777933		UDB0777932
Chironominae				
Afrozavrelia kribiensis	GenBank	HQ440707	HQ440557	
Chironomus sp.	GenBank	HQ440719	HQ440572	
Cladotanytarsus sp. 1	GenBank	HQ440720	HQ440573	
Cladotanytarsus sp. 2	GenBank	HQ440721	HQ440574	
Micropsectra sp.	GenBank	HQ440775	HQ440621	
Paratanytarsus sp. 1	GenBank	HQ440803	HQ440646	
Paratanytarsus sp. 2	GenBank	HQ440804	HQ440647	
Rheotanytarsus sp.1	GenBank	HQ440825	HQ440666	
Rheotanytarsus sp. 2	GenBank	HQ440826	HQ440667	
Stempellina sp. 1	GenBank	HQ440834	HQ440674	
Stempellina sp. 2	GenBank	HQ440835	HQ440675	
Tanytarsus sp.	GenBank	HQ440846	HQ440686	
Diamesinae				
Harrisonina petricola	GenBank	GU356714	GU356732	
Heptagyia annulipes	GenBank	HQ440757	HQ440606	
Limaya sp.	GenBank	HQ440767	HQ440614	
Mapucheptagyia brundini	GenBank	HQ440772	HQ440618	
Paraheptagyia tonnoiri	GenBank	GU356716	GU356734	
Orthocladiinae				
Acricotopus lucens	GenBank	AJ586562	AJ586561	
Acricotopus longipalpus	BOLD	ACRIC-2L-1M		
Cricotopus sp. 1	GenBank	HQ440727	HQ440580	
Cricotopus sp. 2	GenBank	HQ440728	HQ440581	
Limnophyes sp.	GenBank	HQ440769	HQ440615	

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TABLE S2. (Continued)

	Database	28S	18S	COI
Orthocladius (*Euorthocladius*) *luteipes*	GenBank	HQ440784	HQ440631	
Parametriocnemus sp. 1	GenBank	HQ440800	HQ440643	
Parametriocnemus sp. 2	GenBank	HQ440801	HQ440644	
Parapsectrocladius acuminatus	GenBank	HQ440802	HQ440645	
Paratrichocladius sp.	GenBank	HQ440805	HQ440648	
Prodiamesinae				
Monodiamesa sp.	GenBank	HQ440777	HQ440623	
Odontomesa sp.	GenBank	GU356715	GU356733	
Telmatogetoninae				
Telmatogeton japonicus	GenBank	GU356720	GU356738	
Telmatogeton mcswaini	GenBank	GU356721	GU356739	
Thalassomyia frauenfeldi	GenBank	HQ440847	HQ440687	
Outgroup				
Forcipomyia brevipennis	GenBank	HQ440748	HQ440599	